Evaluation of denaturing high performance liquid chromatography for the mutational analysis of the *MEN1* gene

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Abstract

The identification of mutations in the *MEN1* gene causing MEN1 has represented a challenge since the cloning of the gene in 1997 because of the lack of mutation hot-spots in the gene and the lack of phenotype-genotype correlations. The use of denaturing high performance liquid chromatography (DHPLC), a high throughput, reliable and automated heteroduplex-based technique, is the ideal for mutation detection in *MEN1*. In this work, DHPLC was optimised for the screening of the nine coding exons and splice junctions of *MEN1*. Thanks to collaboration between two French laboratories recognised as reference centres for genotypic MEN1 diagnosis (Lyon and Lille), a blind retrospective study conducted in a cohort of 160 unrelated MEN1 probands with (or without) known germline mutations was undertaken to evaluate the sensitivity of DHPLC. We were able to detect 101 different sequence variations by DHPLC, distributed in the 10 analysed DNA fragments and corresponding to 100% of mutation detection compared with direct sequencing. 1.2% of samples were considered as false positive, exhibiting a heterogenous profile. DHPLC did not detect five cases of deletion or duplication of complete exons, neither did direct sequencing, showing the limits of the technique. Nevertheless, the method appeared to allow automated, rapid and low-cost mutation detection with high accuracy. Direct sequencing can be then applied to identify the sequence variations on the targeted DNA fragments showing heterozygous profile by DHPLC. In conclusion, genotypic diagnosis of *MEN1* can benefit from DHPLC in terms of efficacy, rapidity and cost.

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Introduction

High sensitivity in detection of DNA sequence variations is fundamental for mutation analysis in disease associated genes. Well established techniques for mutation detection range from relatively simple methods called scanning methods, e.g. single strand conformation polymorphism (SSCP) and heteroduplex analysis (Crépin *et al.* 2003) to more complex procedures (also as scanning methods or as specific methods), e.g. direct sequencing, protein truncation test, denaturing gradient gel electrophoresis (DGGE) and enzymatic or chemical cleavage methods (Tchernitchko *et al.* 1999, Nedelcheva *et al.* 2001). However, the most sensitive methods are often very labour intensive and expensive. Ideally, the

methods used for mutation analysis of a large number of DNA fragments should be sensitive, non hazardous, relatively inexpensive and fully or at least semi-automated to minimise time and later costs. Rapid turn over time is also of obvious importance for clinicians and their patients. These criteria appear to be met to a high degree for the method called denaturing high performance liquid chromatography (DHPLC; Xiao & Oefner 2001). This technique is based on the detection of heteroduplexes in PCR products by ion-pair reverse phase high performance liquid chromatography (Oefner & Underhill 1998). One of the advantages of this method is the use of automated instrumentation, leading to a high speed analysis. DHPLC has been successfully used for the detection of mutation of several genes

associated with diseases such as *NF1* (O'Donovan *et al.* 1998), *BRCA1* and *BRCA2* (Wagner *et al.* 1999); *hMLH1* and *hMSH2* (Holinski-Feder *et al.* 2003), *VHL* (Klein *et al.* 2001).

One of these genes for which mutations predispose to familial cancers is *MEN1*. The *MEN1* gene is located on chromosome 11q13 and spans 9 kb. It was cloned in 1997 by Chandrasekharappa *et al.*, and the European Consortium on *MEN1* (1997). Deleterious heterozygous mutations of this oncosuppressor gene predispose to Multiple Endocrine Neoplasia type 1 (*MEN1*), which is an autosomal dominant inherited tumour syndrome (OMIM 131100) characterized by tumours of parathyroid, entero-pancreatic neuroendocrine tissue and anterior pituitary (Pannett & Thakker 1999, Schussheim *et al.* 2001). More than 300 deleterious germline mutations are scattered throughout the coding region of the gene and no obvious hot-spot has been identified (Giraud *et al.* 1998, Agarwal *et al.* 2004). There is currently no evidence of genotype/phenotype correlations (Marx *et al.* 1999, Wautot *et al.* 2002). As a consequence, all the expressed *MEN1* exons, including exon/intron boundaries, need to be screened for mutation detection. For these reasons, a sensitive automated approach for mutation scanning was desirable. Owing to the above mentioned advantages of using DHPLC, we decided to evaluate the sensitivity and specificity of DHPLC as a first intention method for *MEN1* mutation analysis in a population of patients.

Materials and methods

Patients

With the aim of evaluating DHPLC as a screening mutation technique, a blind retrospective study on 160 anonymised DNA samples was undertaken (in Lille). The samples were obtained from non-related French patients with *MEN1* syndrome, previously screened for *MEN1* mutations by direct sequencing (in Lyon). All patients gave their informed consent for the genetic analysis. Genomic DNA was extracted from whole venous blood with QIAAMP DNA blood Maxi kit (Qiagen, Courtabeuf, France) following the manufacturers' instructions. Additional DNA samples from affected and non affected patients were used as positive and negative controls, respectively. They were collected and prepared using the same conditions.

PCR amplification

The coding sequence of *MEN1* (exons 2–10) was amplified from purified DNA using the primers previously described by Crépin *et al.* (2003) and selected from the sequence published in 1997 by Chandrasekharappa *et al.* (U93237.1). Exons 2 and 10 were

amplified in two partially overlapping DNA fragments and exons 5 and 6 together. This was necessary to homogenise the size of the DNA fragments ranging from 192 to 369 bp.

Amplifications were carried out by PCR in a 50 µl reaction volume containing 25 pM of each primer, 0.5 U of Hot Star Taq polymerase (Qiagen) and 75 ng of genomic DNA. Amplifications were performed with the following 'touchdown' PCR conditions: initial denaturation at 94 °C for 12 min; 9 cycles of: denaturation at 95 °C for 30 s, annealing with a decrease of 1 °C/cycle from 63 to 55 °C for 90 s, primer extension at 72 °C for 90 s; 26 cycles of: 30 s at 95 °C, 90 s at 55 °C, and 90 s at 72 °C; then a final elongation step at 72 °C for 5 min.

DHPLC analysis

Blind DHPLC analysis was performed on a wave DNA fragment analysis system (Transgenomic, San Jose, CA, USA). Each PCR product was denaturated for 5 min at 95 °C and then gradually re-annealed by decreasing the sample temperature from 95 to 25 °C (with a temperature ramp of 1.75 °C/min) over a period of 40 min. Two to five µl of PCR product was then applied to a pre-heated C18 reversed-phase column based on non porous poly(styrene-divinylbenzene) particles (DNASep, Transgenomic) and eluted with a mobile phase consisting of a mixture of 0.1 M triethylammonium acetate pH 7.0 (TEAA) (Transgenomic) and 0.1 M TEAA with 25% acetonitrile (Transgenomic) in a linear gradient, at a flow rate of 1.5 ml/min. Gradient parameters were determined by size and GC content of the amplicons. Heteroduplexes and homoduplexes were detected by monitoring the absorbance at 260 nm.

The condition for mutation analysis is based on the melting behaviour of the wild type sequence of the DNA fragment (Jones *et al.* 1999) rather than by its length. Temperature for successful resolution of heteroduplex molecules was determined by using the WaveMaker software (Transgenomic) and the Stanford DHPLC program (available at: www.insertion.stanford.edu/melt.html). From the sequence of each fragment, the algorithm calculates the melting behaviour, the optimal analysis temperature for each domain corresponding to 80–90% of α -helical fraction. During the optimisation phase, a few mutated samples for each DNA fragment were additionally run up to 2 °C above and below of the predicted temperatures, with 0.1 °C increments, to select the optimal temperatures for detection of sequence variations. Most of the exons required two to three temperatures for mutation analysis due to the different melting domains in the corresponding DNA fragment. Thus the softwares indicated a mean temperature for mutation detection but adjustment was necessary because some sequence variations were difficult to

Table 1 Temperatures selected for DHPLC analysis for each DNA fragment

Exon/ (DNA fragment)	Fragment size (bp)	Analysis temperatures
2/(2AB)	353	65.1 °C/67.6 °C
2/(2CD)	263	60.4 °C/61.9 °C/63.4 °C
3	305	62.3 °C/64.5 °C
4	192	60.9 °C/62.5 °C
5+6	297	60.2 °C
7	250	61.5 °C/64 °C
8	217	62.1 °C
9	245	61.6 °C/64.1 °C
10/(10AB)	369	67.2 °C
10/(10C)	253	62.8 °C

visualise. The selected analysis temperatures are summarised in table 1.

DNA sequencing

As the study was retrospective, all of the 160 DNA samples had already been analysed by direct sequencing of the whole coding region of *MEN 1*, using 100 ng of purified DNA product and an ABI PRISM Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystems, Courtaboeuf, France). Sequencing was performed on the ABI PRISM 377 DNA sequencer (Applied Biosystems). Each DNA fragment harbouring a modified DHPLC pattern compared with the negative control was further analysed by sequencing.

Results

The conditions for mutation analysis of the various DNA fragments were determined on the basis of the data obtained with a few mutated samples with previously known sequence variations. For the study, 160 DNA samples were analysed for the 10 DNA fragments covering the coding exons of *MEN1* (so representing 1600 DNA fragments). Due to the insufficient amount of DNA for some samples, only 1473/1600 DNA fragments could be amplified (92%).

The analysis of one DNA sample was completed in a few hours which include PCR, generation of heteroduplexes, and elution of each of the 10 DNA fragments at one to three different temperatures.

DHPLC analysis enabled us to detect 213 DNA fragments with sequence variation, corresponding to 101 different sequence variations covering all the tested DNA fragments. These are listed in table 2. For most cases, detection of abnormal elution profiles was

performed at the two or three selected temperatures, comparing to a negative control profile. Nevertheless, for some of them located on specific melting temperature domains, only one tested temperature revealed a heterogenous profile, justifying to select multiple temperatures for analysis. (e.g: c88–16C>G; c1364C>T).

As it was a retrospective blind analysis, all these sequence variations had already been identified by sequencing. These have been recorded in the French GTE database (Groupe d'étude des Tumeurs Endocrines; <http://www.fichiergte.com>) and also most of them are in international databases such as HGMD (Human Gene Mutation Database) (<http://uwcmmls.uwcm.ac.uk.html>).

Among these sequence variations, some corresponded to known polymorphisms. We found them with a frequency close to the data published, i.e.: c88–16C>G: 16/120 (13.33%); c545C>T (pS145S): 11/149 (7.38%); c622 G>A (pR171Q): 4/151 (2.65%); c1364C>T (pD418D): 65 /141(46.1%); c1731 G>A (pA541T): 6/ 151 (3.97%). Few other sequence variations, not recorded as polymorphisms, were detected in more than one sample. It is noticeable that virtually each sequence variation leads to a specific elution profile and that the same sequence variation in different samples leads to the same profile, displaying a good reproducibility. Figures 1 and 2 show few examples of mutation profiles in different DNA fragments (compared with negative controls).

No sequence variation was missed by DHPLC analysis, except the five samples with deletions or duplications of complete exons, which do not lead to the promotion of heteroduplexes and which are also missed by sequencing. Only semi-quantitative methods, such as quantitative multiplex PCR of short fluorescent DNA fragments (QMPSP; Casilli *et al.* 2002) are able to assess exon copy number and detect this type of mutation. Such methods are performed when *MEN1* syndrome is clinically evident and no *MEN1* mutation is found by conventional methods. Thus, the sensitivity of the method can be considered to be 100% compared with sequence analysis (213/213 'positive' DNA fragments).

Some DNA fragments (18) exhibited heterogenous profiles (compared with the negative controls) without corresponding to any sequence variation, especially in the DNA fragments corresponding to exon 8. This could be due to the DNA quality or to the complexity or the heterogeneity of these DNA fragments. Out of 1260 negative DNA fragments by sequencing, 1242 showed a 'negative' DHPLC profile and 18 could be considered as false positive (if sequencing is the reference method). As a consequence, the specificity of the method can be considered to be 98.6% (1242/1260) due to these 18 doubtful DNA fragments which can be considered as 'false positive'.

Table 2 Tabulation of 101 sequence variations detected by DHPLC

Location	Sequence variation	Number of samples
int1	c88-16C>G	(P) 16
ex2	c117del	1
ex2	c177del	1
ex2	c195C>T	1
ex2	c223C>G	1
ex2	c255G>C	1
ex2	c285del	1
ex2	c306_310dup	4
ex2	c318_319insGGTG	1
ex2	c337del	1
ex2	c357_360del	1
ex2	c359_363del	1
ex2	c382del	2
ex2	c396C>T	1
ex2	c402C>T	1
ex2	c432C>T	1
ex2	c437_438insGG	1
ex2	c464_466del	1
ex2	c485_486del	1
ex2	c509C>A	1
ex2	c523del	1
ex2	c541T>G	1
ex2	c545C>T	(P) 11
int2	c556-3C>G	1
ex3	c562_563del	2
ex3	c576G>T	1
ex3	c577G>A	1
ex3	c581G>A	(P) 1
ex3	c603T>C	1
ex3	c622G>A	(P) 4
ex3	c644insT	1
ex3	c645G>C	1
ex3	c699_701del	1
ex3	c710_711insCA	1
ex3	c711_712dup	1
ex3	c738_741del	1
ex3	c747del	1
ex3	c764G>T	1
int3	c765-6C>A	1
int3	[c765-6C>G;c765-3A>G]	1
int3	c765-6C>T	1
ex4	c776del	1
ex4	c778T>C	2
ex4	c804C>T	1
ex4	c883insA	1
ex5	c905G>A	1
ex5	c924insC	1
ex5	c934G>A	2
int5	c934+5G>A	1
int5	c934+35G>A	1
ex6	c951G>A	2
ex6	c1016_1017del	1
ex7	c1041A>C	1
ex7	c1049T>G	1
ex7	c1051G>C	1
ex7	c1077T>C	1
ex7	c1079C>G	1
ex7	c1120C>A	2
ex7	c1131T>C	1
ex7	c1155C>T	1

Table 2 Continued

Location	Sequence variation	Number of samples
int7	c1159+1G>A	1
int7	c1159+2T>C	1
ex8	c1170del	1
ex8	c1179G>C	1
ex8	c1213C>A	1
ex8	c1228C>G	1
ex9	c1298_1301del	1
ex9	c1325del	1
ex9	c1353C>T	1
ex9	c1362G>A	1
ex9	c1364C>T	(P) 65
ex9	c1374del	1
ex9	c1381insG	1
ex9	c1384_1386del	1
ex9	c1409C>T	(P) 2
ex9	c1418G>A	1
ex9	c1424C>G	1
int9	c1460+1_1460+11del	2
int9	c1460+4del8insGTCTGT	1
int9	c1461-1G>T	1
ex10	c1486_1493dup	1
ex10	c1486_1508del	2
ex10	c1487delCinsACATAGT	1
ex10	c1488C>T	3
ex10	c1489ins17	1
ex10	c1490_1501del12ins5	1
ex10	c1583insC	1
ex10	c1593insC	1
ex10	c1634del	1
ex10	c1650del	1
ex10	c1650insC	2
ex10	c1656insG	1
ex10	c1689C>T	1
ex10	c1699del	1
ex10	c1716C>T	1
ex10	c1728C>T	1
ex10	c1731G>A	(P) 6
ex10	c1774G>C	1
ex10	c1774G>A	1
ex10	c1780_1782del	1
ex10	c1782del	1
		213

Nomenclature according to HGVS recommendations; numbering based on *MEN1* cDNA sequence (accession number U93236) (P), polymorphism

In case of any 'positive' DHPLC result (detection of abnormal profile in comparison to negative control), the sequence analysis remains essential for confirming and identifying the sequence variation.

Discussion

DHPLC mutation analysis for the *MEN1* gene carried out in 160 French patients was rapid and sensitive. Standardisation of PCR conditions and semi-automation

Figure 1 Representative DHPLC patterns in *MEN1* DNA fragments (exon 2 to exon 4). From top to bottom and left to right, respectively, ex 2AB (67.6 °C); ex 2CD (61.9 °C); ex 3 (64.5 °C); ex 4 (62.5 °C).

of methodology allowed high throughput of analysis. We were able to detect 101 different sequence variations in *MEN1*, yielding a sensitivity of 100% compared with sequence analysis. Whatever the great heterogeneity of mutations responsible for *MEN1* was, this blind analysis allowed us to conclude that the running conditions previously defined for each DNA fragment were optimised.

Specificity greatly depends on the purity of the PCR products. If the DHPLC instrument is used in carefully monitored conditions, according to robust standard operating procedures, this technique offers a high reproducibility. The previously used mutation screening strategy in our laboratory for *MEN1* (Crépin *et al.* 2003) required the combination of the two techniques (MDGA

and HMA) to yield 100% sensitivity and was therefore much more time consuming and expensive. Marsh *et al.* (2001) and Roberts *et al.* (2001) showed that DHPLC is well adapted to rapid mutation scanning. Due to frequent polymorphisms, especially in exon 9, DHPLC patterns could hide another mutation in the polymorphic DNA fragments. In these cases, DNA sequencing remained essential. Nevertheless, the mean rate of sequencing for *MEN1* remained much lower than without use of DHPLC as a screening method. Consequently, the cost of *MEN1* genotypic diagnosis was estimated as being five times cheaper with this strategy, compared with complete sequencing of the gene. This is in accordance with Sevilla *et al.* (2002) who made an economic analysis of *BRCA1* testing by different

Figure 2 Representative DHPLC patterns in *MEN1* DNA fragments (exon 5 to exon 10). From top to bottom and left to right, respectively, ex 5+6 (60.2 °C); ex 7 (64.0 °C); ex 8 (62.1 °C); ex 9 (64.1 °C); ex 10AB (67.2 °C); ex 10C (62.8 °C).

techniques and showed that DHPLC was by far cheaper than direct sequencing or other screening methods such as SSCP or DGGE.

Virtually each sequence variation led to a specific elution profile. Of course, the optimal resolution should show all four hetero- and homoduplexes fully resolved. In practice some mutations exhibited a better resolution than others because the stability of the heteroduplexes varied and the flanking base pairs created different surroundings (Van den Bosch *et al.* 2000). Therefore, our study allowed us to constitute a pattern database of more than 100 sequence variations for *MEN1*. Nevertheless, patterns need to be interpreted with great caution because in some cases, patterns corresponding to different sequence variation looked very similar to each other (e.g. figure 1, exon 5+6: c905 G>A and c934 G>A). The identification of mutations by sequencing remains essential, especially with *MEN1*, for which new mutations are found regularly.

Some authors showed that DHPLC was also able to detect a low-level of mosaicism (Emmerson *et al.* 2003, Hendy *et al.* 2003). It can thus be hypothesized that mosaicism could be underestimated for most genotypic analysis, because of the difficulty of detection by conventional methods. Takashima *et al.* (2001) estimated

that DHPLC may perform better than conventional automated DNA sequence analysis for patient mutation detection. They described a number of sequence variations, not detected by direct DNA sequencing, that were detected by DHPLC. The collection of DHPLC heteroduplex fractions enabled us to subsequently identify the corresponding sequence variations.

DHPLC could also be applied to detection of somatic mutations in tumours, as shown by Liu *et al.* (1998), Lu *et al.* (2003) with *p53* and *ST7* genes in gastric carcinomas, or Gross *et al.* (1998) with *p53* in ovarian tumours. In addition, the authors also used frequent polymorphisms to rapidly identify loss of heterozygosity (LOH) in tumours, which is usually part of the mechanism encountered for tumour suppressor genes such as *p53* but also *MEN1* (Pannett & Thakker 2001).

Whatever the performances of DHPLC, the characteristics of the method make the detection of complete deletion or duplication of exons impossible. However, SSCP, DGGE or sequence analysis exhibit the same limitations. These methods are therefore unsuitable for comprehensive mutation detection in diseases known to be associated with exon deletions or duplications as main mutational mechanism. Semi-quantitative methods are then needed to detect these large rearrangements. An

elegant multiplex ligation-dependent probe assay has been recently described, related to the previously described multiplex amplifiable probe hybridization (Armour *et al.* 2000, Schouten *et al.* 2002). Lastly, various semi-quantitative multiplex fluorescent PCR protocols have been used to detect large rearrangements culminating in the so-called QMPFS (Casilli *et al.* 2002). Also more recently, some authors described a new, versatile and robust method to assess exon copy number, called multiplex PCR/liquid chromatography assay (Dehainault *et al.* 2004). Large deletions or duplications can also be assessed by high resolution comparative genome hybridization microarrays methodology (Porchet *et al.* 2004).

Mutational analysis of *MEN1* achieved by complete sequence analysis of the nine coding exons and corresponding exon/intron boundaries is expensive and time consuming. It is clear that DHPLC is a reliable and economic way to screen out the DNA fragments of wild-type sequence and select only those showing evidence of sequence variations for nucleotide sequencing. This facilitates a broad genotypic screening in routine analysis of *MEN1* in patients for whom the diagnosis of *MEN1* is evoked on few clinical or biological arguments (for example hyperparathyroidism alone occurring at a young age), for early detection and treatment. It can also be useful for differential diagnosis because the clinical features of this disease are diverse. Park *et al.* (2003) screened for *MEN1* mutations, by DHPLC, five *MEN1* typical families but also one family with familial isolated hyperparathyroidism and one family with familial pituitary adenoma. Recent studies show that mutation testing for *MEN1* remains justified for all patients with clinical typical criteria of *MEN1* and also for isolated hyperparathyroidism cases (familial cases or sporadic cases in young people) (Cardinal *et al.* 2005). DHPLC takes up the challenge of extending the screening for other genes involved in neighbored syndromes, as done by Biasiotto *et al.* (2003) for hereditary hemochromatosis. Therefore, the phenotypic heterogeneity of the disorder could be grasped and genotype–phenotype correlations could be defined.

In conclusion, we propose a DHPLC protocol which was found to be reliable, cost effective, rapid, sensitive and specific for *MEN1* mutation screening. Large genetic testing studies on *MEN1* have recently been published (Cardinal *et al.* 2005, Ellard *et al.* 2005, Klein *et al.* 2005) but all were based on PCR sequencing of all the coding regions of *MEN1*. To our knowledge, it is the first time that the *MEN1* gene has been evaluated on such a large number of samples by a screening method with 100% sensitivity, therefore allowing its use in routine analysis and avoiding systematic complete sequencing of the gene. DHPLC has become a standard pre-screening tool for mutation detection and Schollen

et al. (2005) recently described a collaborative work from diagnostic laboratories with DHPLC expertise to develop standard operating procedures for quality assurance of molecular diagnostic tests performed with this system. In the same way, our study allows other laboratories to directly perform DHPLC mutation screening of the *MEN1* gene with a robust validated method.

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